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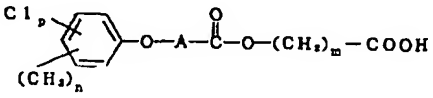
(54) Title of the Invention: Hapten compound of phenoxyacetic acid, antibody and assay thereof

(57) [Abstract of the Disclosure]

[Object] The object of the present invention is to provide a hapten compound of phenoxyacetic acids, antibody and an assay thereof.

[Construction] An antibody of the present invention is produced by using phenoxyacetic acid itself or phenoxyacetic acid derivatives represented by the following formula I as a hapten:

[Compound 1]



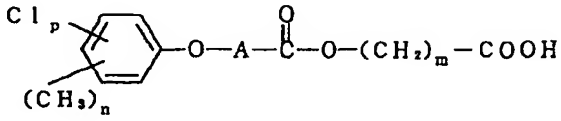
Formula I

wherein A is an alkylene group having 1-3 carbon atoms which may be branched, m is an integer of 1-10, n and p are an integer of 0-3.

[Claims]

1. A compound having a structure represented by the following formula:

[Compound 1]



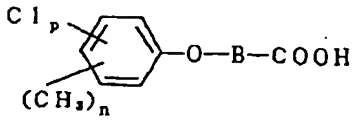
Formula I

wherein A is an alkylene group having 1-3 carbon atoms which may be branched, m is an integer of 1-10, n and p are an integer of 0-3.

2. A conjugate of the compound of formula I and a high molecular compound.

3. A method of producing an antibody or a fragment thereof exhibiting a reactivity to a compound represented by the formula II, characterized in that a high molecular compound is bound to the compound of the formula I to produce an antigen, and the antigen is used to produce the antibody exhibiting a reactivity to the compound represented by the formula II:

[Compound 2]



Formula II

wherein B is selected from the group consisting of a methylene group, an ethylidene group and a trimethylene group, and n and p are as defined in the Formula I.

4. An antibody or a fragment thereof exhibiting a reactivity to a compound of the Formula II, which is produced by using a conjugate of the compound of the Formula I or the Formula II and a high molecular compound as an antigen.

5. The antibody or fragment thereof of claim 4, wherein in the compound of the Formula I or the Formula II, a benzene ring is unsubstituted, or substituted by a substituent selected from the group consisting of 3-chloro, 4-chloro, 2,4-dichloro, 2,4,5-trichloro, and 4-chloro-2 methyl; and A is selected from the group consisting of a methylene group, an

ethylidene group, and a trimethylene group.

6. The antibody or fragment thereof of claim 4 or 5, wherein the antibody is a monoclonal antibody.

7. The antibody or fragment thereof of any one of claims 4 to 6, having a reactivity to the compound of the Formula II in methanol of 40% or less.

8. The antibody or fragment thereof of any one of claims 4 to 7, having a reactivity to 2,4,5-trichlorophenoxyacetic acid and 3,5,6-trichloro-2-pyridyloxyacetic acid.

9. The antibody or fragment thereof of any one of claims 4 to 8, wherein the antibody is TCA28-50.

10. A hybridoma producing an antibody or a fragment thereof according to any one of claims 4 to 9.

11. The hybridoma of claim 10, wherein the hybridoma is deposited in a accession number of FERM P-15848.

12. An immunoassay of the compound represented by the Formula II, characterized in that an antibody or a fragment thereof according to any one of claims 4 to 9 is used.

13. The immunoassay of claim 12 comprising, further using at least one compound selected from the Formula I or the Formula II as a label compound.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to hapten compounds of phenoxyacetic acid derivatives and phenoxybutyric acid derivatives (hereinafter referred to as "phenoxyacetic acids"), antigens, antibodies and fragments thereof.

[0002]

The present invention further relates to an immunoassay using the above antigens, antibodies and fragments thereof.

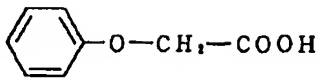
[0003]

[Prior Art]

Phenoxyacetic acids are a series of compounds of phenoxyacetic acid

represented by the formula:

[Compound 3]

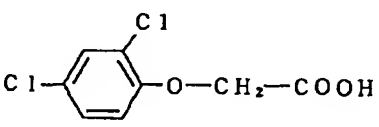


in which a phenyl group is substituted. The phenoxyacetic acids include derivatives such as salt or ester of phenoxy acids; a series of compounds in which α position of the derivatives is substituted;, and a series of compounds of similar derivatives of 4-phenoxybutyric acid. Many of them are widely used as herbicides including 2,4 PA agent (2,4-D), MCP agent, MCPB agent, MCPP agent, and similar agents of trichlopyr, phenothiol, chlomeprop agent, and naproanilide. These herbicides act on a cell membrane at a low concentration in similar manner to ingrown auxin (IAA) in plant body, to cause the structural change, promote the biosynthesis of DNA-RNA-protein by activation of enzyme and function as a plant growth regulator leading to looseness of cell wall - absorption of water - growth of cell. However, at high concentration, it is thought that they do not regulate metabolism, thereby they disturb the function of plant hormones to become death of plants. Broad-leafed weed is more susceptive than grass weed, because it is said that it has more migration and absorption from stems and leaves, and particularly in growth point the concentration of the herbicides becomes high. Rice has a high susceptibility at the germination, so that transplanted rice sometimes causes etiolation and tubular leaf according to temperature and amount of herbicides (Pesticide handbook, p.318-323, 1994 edition, Japan Plant Protection Association).

[0004]

A representative of phenoxyacetic acids is 2,4-dichlorophenoxyacetic acid (hereinafter referred to as 2,4-D) represented by the formula.

[Compound 4]



2. 4-D

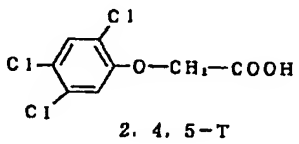
2,4-PA agent containing 2,4-D as an active ingredient is a hormone type

selective herbicide and kills broad-leaved weed without giving a serious chemical damage to grass crop. When the 2,4-PA agent is spread to broad-leaved weed, it is absorbed from stems and leaves, and migrated in a plant body, so that it acts on meristematic tissues as 2,4-PA acid to cause an abnormality of meristematic tissue and disturbance of physiology, thereby leading to tortional deformity, decomposition of root base and inhibition of root growth, finally to kill the weed. It is said that the agent is easily shifted in soil and its effective maintaining period is about 20 days in summer.

[0005]

2,4,5-Trichlorophenoxyacetic acid (hereinafter referred to as 2,4,5-T) represented by the formula:

[Compound 5]



2, 4, 5-T

has a similar action to 2,4-D. It has been developed as a herbicide for forest because it has greater effect to shrubs than 2,4-D. 2,4,5-T is a hormone type selective herbicide having transferability. It has higher activity in trees and shrubs than plants, and in high temperature conditions has great effects to both treatments of stems and leaves and also soil. It has been produced in an agent such as sodium salt, potassium salt, ammonium salt, amine salt and alkyl esters, in Japan, mainly butoxyethyl ester (brand name: Wyedon) has been commercialized. It was registered as a pesticide during September 1964 and April 1975, but now it is prohibited in use. Additionally, as is described in the reference listed in the present specification, ADI of 2,4,5-T is 0.03 mg/kg. (Reference: Recent analysis of residual pesticides, p.21-23, edited by Research group of analysis of residual pesticides, Cyuohouki Publishers).

[0006]

In recent years, there are a lot of strong concerns about residual pesticides in soil, water and air, and postharvest pesticides of imported farm

products that are particularly increasing recently. For example, regarding the 2,4,5-T, it is required that 2,4,5-T showed not be detected in rice, wheat, miscellaneous grains, fruit, vegetables, potatoes, beans, seed, tea and hop (above described reference: Recent analysis of residual pesticides). In order to ensure safety on environments and foods, it is necessary to measure phenoxyacetic acids with high speed and high accuracy.

[0007]

Conventionally, phenoxyacetic acids are extracted from a sample of rice, fruit or potatoes, and after purification, it is analyzed by a gas chromatography (GC). For example, in the case of 2,4,5-T, an assay is performed by extracting a sample with acetone, dissolving the extract in ethyl acetate and hexane respectively, followed by eluting with florisil column chromatography and further analyzing by GC (above described reference: Recent analysis of residual pesticides). There are problems in such an analysis that sample preparation is complicated and needs a lot of labor and time, skill in analysis, and large expenses in measuring apparatus and facilities. An assay of phenoxyacetic acids, particularly in an analysis of residual pesticides of imported farm products, needs a vast number of analysis results for samples in a short time. It has been demanded to develop a new measuring method of phenoxyacetic acids having not only precision but also simplicity, high speed and economical efficiency.

[0008]

Immunoassay is a method where an antibody recognizes an antigen specifically, the antigen is detected by antigen-antibody reaction, and it has been recently attracted from the points of excellent precision, simplicity, high speed and economical efficiency. In the immunoassay, there have been applied extremely various kinds of labels for the assay, for example, enzyme, radioactive tracer, chemiluminescence or fluorescent substance, metal atoms, sol, a stable free radical, latex and bacteriophage.

[0009]

Among immunoassays, enzyme immunoassay (EIA) has been widely used as an excellent method. The excellent reviews on enzyme

immunoassay method are described in "Practice and theory of enzyme immunoassays in Laboratory techniques," by Tijssen P, in Biochemistry and molecular biology, Elsevier Amsterdam New York, Oxford ISBN 0-7204-4200-1 (1990).

[0010]

Generally, by injecting a molecule with high molecular weight to animals without further modification, a suitable immune reaction is induced, so that an antibody recognizing an antigen can be produced. However, low molecular compounds such as phenoxyacetic acids cannot induce an immunologic reaction when they are normally injected in animals. Only after these molecules are bound with a high molecular weight compound having immunogenicity, they act as a group of epitope, and cause an immune reaction in the presence of T cell accepter, as a result, an antibody is produced by a group of B lymphocytes. In this way, a molecule generating immunogenicity only after being bound with a high molecular compound is generically called as a hapten.

[0011]

Further, when a low molecular compound is bound with a high molecular compound to generate an antigen, it is also effective that a low molecular compound that is introduced with a spacer arm (binding arm) together with functional groups available to binding is used as a hapten. However, in this case, if the introduction with consideration of all problems such as disposition of binding arm/functional group, size of binding arm is not suitable, no desired antibody is obtained. Suitable introduction should be devised depending on respective molecules.

[0012]

Although the need of phenoxyacetic acids is very high, no suitable antibody has been obtained. Additionally, as a hapten in the production of antibody, no phenoxyacetic acid derivative in which a spacer is bound with phenoxyacetic acids was obtained before the present invention.

[0013]

[Problems to be Solved by the Invention]

The object of the present invention is to provide phenoxyacetic acid derivatives which are hapten compounds constituting an antigen that is used to produce a new antibody that reacts specifically with phenoxyacetic acids.

[0014]

The object of the present invention is also to provide a conjugate of phenoxyacetic acids or phenoxyacetic acid derivatives in which the phenoxyacetic acids is bound with a spacer and a high molecular compound. The conjugate becomes an antigen to produce an antibody that reacts specifically with phenoxyacetic acids.

[0015]

The object of the present invention is further to provide a new antibody or fragment thereof reacting with phenoxyacetic acids, and a producing method thereof.

[0016]

An embodiment of the present invention provides a monoclonal antibody having a reactivity to 2,4,5-T and 3,5,6-trichloro-2 pyridyloxyacetic acid (hereinafter referred to as trichlopyr). The antibody is characterized by recognizing 2,4,5-T in concentration dependence in presence of methanol of 40% or less.

[0017]

Furthermore, the object of the present invention is further to provide a hybridoma producing the antibody and fragment thereof.

[0018]

Further, the object of the present invention is further to provide an immunoassay of phenoxyacetic acids including the use of the antibody.

[0019]

The term "fragment" in the present specification means a portion of antibody capable of binding with an antigen, F_{ab} fragment, for example.

[0020]

[Means for Solving the Problems]

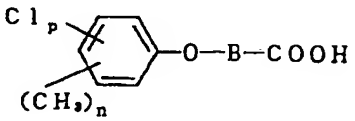
The present inventors conducted in-depth research on the above

described problems, as a result, they succeeded in obtaining a specific antibody to phenoxyacetic acids by using phenoxyacetic acids itself or phenoxyacetic acid derivatives in which the phenoxyacetic acids is bound with a spacer as hapten, and have completed the present invention. Namely, in the present invention, an object phenoxyacetic acids itself can be used as the hapten and bound with a high molecular compound to produce an antigen, or phenoxyacetic acid derivatives in which the above-described phenoxyacetic acids bound with the spacer can be used as the hapten.

[0021]

An object phenoxyacetic acid of the present invention is a compound having a structure represented by the following formula II:

[Compound 6]



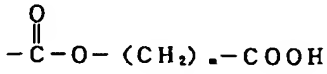
Formula II

wherein B is selected from the group consisting of a methylene group, an ethylidene group and a trimethylene group, and n and p are an integer of 0-3. In a chemical structure in the present specification, a phenyl group may be optionally a pyridyl group.

[0022]

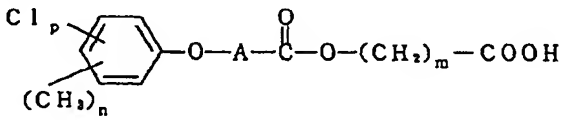
When phenoxyacetic acid derivatives are used as hapten, -COOH in the phenoxyacetic acids is changed to the formula:

[Compound 7]



wherein m is an integer of 1-10, preferably 3. Namely, phenoxyacetic acid derivatives of the present invention are a compound having a structure represented by the formula I:

[Compound 8]



Formula I

wherein A is an alkylene group having 1-3 carbon atoms which may be branched, n and p are defined in the Formula II, and m is an integer of 1-10, preferably 3.

[0023]

It is not intended to limit, particularly preferable compound is, in Formula I or Formula II, benzene ring is un-substituted, or substituted by a substituent selected from the group consisting of 3-chloro, 4-chloro, 2,4-dichloro, 2,4,5-trichloro, and 4-chloro-2 methyl; and A is selected from the group consisting of a methylene group, an ethylidene group, and a trimethylene group.

[0024]

A specific antibody to phenoxyacetic acids can be obtained by using a hapten as an antigen, the hapten being the phenoxyacetic acids or phenoxyacetic acid derivatives bound to the spacer of the present invention which are bound with a suitable high molecular compound.

[0025]

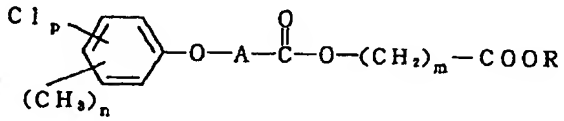
The present invention relates to the hapten compound, a conjugate of hapten compound and high molecular compound, an antibody reacting to phenoxyacetic acids, a production method thereof, and an immunoassay of antibody phenoxyacetic acids using the hapten compound or the antibody.

[0026]

Preparation of phenoxyacetic acid derivatives

The phenoxyacetic acid derivatives represented by the Formula III can be produced by a known method. For example, it can be produced from esters represented by the Formula III:

[Compound 9]



Formula III

wherein R represents a protective group for a carboxylic group, A, n, p and m are defined in Formula I, by removing a protective group for a carboxyl

group represented by R.

[0027]

In the above Formula III, a protective group for a carboxyl group represented by R may be a known group, the illustrative example includes a methyl group, an ethyl group, a tert-butyl group, a benzyl group, a p-metoxybenxyl group, a trichloroethyl group, a trimethylsilyl group, a tert-butyldimethylsilyl group, a tert-butyldiphenylsilyl group, a triethylsilyl group, a triisopropylsilyl group, and a trimethylsilylethoxymethyl group.

[0028]

Removal of the protective group for a carboxyl group represented by R can be carried out by a known method such as acid hydrolysis and alkali hydrolysis.

[0029]

For example, when the protective group is a tert-butyl group, an acidic catalyst is added to an organic solvent containing an ester compound of Formula III to allow to react preferably while stirring. As an organic solvent, for example, aromatic hydrocarbons such as benzene, halogenated hydrocarbons such as dichloromethane and 1,2-dichloroethane, or a mixed solvents thereof can be used. As the acidic catalyst, a known catalyst can be used, including carboxylic acids such as trifluoroacetic acid, sulfonic acids such as p-toluenesulfonic acid, mineral acids such as hydrochloric acid and nitric acid, among them, trifluoroacetic acid is preferred. Reaction temperature is usually 0 to 50°C, preferably room temperature, and reaction time is usually about 0.5 to 3 hours.

[0030]

In the case of alkali hydrolysis, an ester compound of Formula III is preferably dissolved in an organic solvent such as methanol, ethanol, tetrahydrofuran or ethylene glycol, or a mixed solvent of water and the organic solvent, and sodium hydroxide, lithium hydroxide or potassium hydroxide is added thereto, the resultant mixture is reacted at 0°C to a temperature of boiling point of solvent, preferably room temperature to 50°C for 1 to 2 hours while stirring to give a carboxylic acid compound of Formula

I.

[0031]

Further, to remove a benzyl group, catalytic reduction reaction with hydrogen can be performed.

[0032]

Further, a trimethylsilyl group, a tert-butyldimethylsilyl group, a tert-butyldiphenylsilyl group, a triethylsilyl group, a triisopropyl group and a trimethylsilylethoxymethyl group can be removed specifically by a reagent generating a fluorine ion such as hydrogen fluoride, tetrabutylammonium fluoride, pyridinium fluoride.

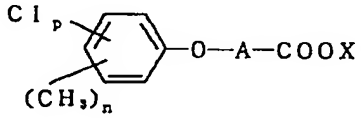
[0033]

In the Formula I or III, m is an integer of 1-10, preferably 3. Here, m means that a reactant is practically represented by an integer.

[0034]

An ester compound of Formula III can be synthesized in various methods. For example, a compound of formula IV:

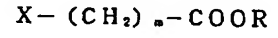
[Compound 10]



Formula IV

wherein X represents a halogen atom (in the specification, halogen atom means F, Cl, Br or I) is reacted with an ester compound of Formula V:

[Compound 11]



Formula V

wherein m is the same as the Formula I, in a solvent in presence of phase-transfer catalyst to give an ester compound of Formula III. The solvent used includes benzene, toluene, xylene, dichloromethane, chloroform, carbon tetrachloride, diethyl ether, tetrahydrofuran, dioxane, acetone, methyl etyl ketone, acetonitrile, ethyl acetate, N,N-dimethylformamide (DMF) and dimethylsulfoxide. A preferable solvent is DMF. A phase-transfer catalyst includes a known catalyst such as crown ethers. A preferable phase-transfer catalyst is 18-crown-6. Reaction temperature is

minus 10 to 100°C, preferably 20 to 30°C, and reaction time is 1 to 48 hours, preferably 5 to 24 hours.

[0035]

By the method above described, a compound is obtained as a crystal. If necessary, it can be treated by a silica gel chromatography or recrystallization to yield a product with higher purity.

[0036]

Hereinafter, it will be described about an antigen of the present invention, a preparation of antibody and an immunoassay. A known method for preparing them can be carried out according to the method described in e.g. Zoku-Seikagaku Jikken Koza, Meneki Seikagaku Kenkyuhou (edited by Japanese Biochemical Society).

[0037]

Preparation of conjugate of phenoxyacetic acids or phenoxyacetic acid derivatives and a high molecular compound

The phenoxyacetic acid derivatives synthesized above or phenoxyacetic acids itself is bound with a high molecular compound, which is used as an immunogen.

[0038]

A preferable example includes keyhole limpet hemocyanin (KLH), egg albumin, bovine serum albumin (BSA), rabbit serum albumin.

[0039]

Binding of phenoxyacetic acids or derivatives thereof with a high molecular compound can be performed by a known method for example, an activated ester method described in A. E. KARU et. al, J. Agric. Food Chem., vol. 42, 301-309 (1994) or by a mixed acid anhydride method in B. F. Erlanger et. al, J. Biol. Chem., vol. 234, 1090-1094 (1954).

[0040]

The activated ester method can be generally carried out as follows; firstly, a hapten compound is dissolved in an organic solvent, the mixture is reacted with N-hydroxysuccinimide in presence of a coupling agent to produce an activated N-hydroxysuccinimide ester.

[0041]

As a coupling agent, al coupling agent commonly used in condensation reaction can be used, and includes dicyclohexyl carbodiimide, carbonyl diimidazole, and water-soluble carbodiimide. As an organic solvent, for example, N,N-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and dioxane can be used. The mole ratio of a hapten compound used for reaction and N-hydroxysuccinimide is preferably 1:10 to 10:1, more preferably 1:1 to 1:10, most preferably 1:1. The reaction temperature is 0 to 100°C, preferably 5 to 50°C, more preferably 22 to 27°C, reaction time is 5 minutes to 24 hours, preferably 30 minutes to 6 hours, more preferably 1 to 2 hours. The reaction temperature can be each chosen at above melting point and below boiling point of each compound.

[0042]

After coupling reaction, the reaction mixture is centrifuged, the resultant supernatant is added to a solution of high molecular compound to allow to react, for example, when the high molecular compound has a free amino group, an acid amide bonding is generated between the amino group and a carboxyl group of the hapten compound. The reaction temperature is 0 to 60°C, preferably 5 to 40°C, more preferably 22 to 27°C, and the reaction time is 5 minutes to 24 hours, preferably 1 to 16 hours, more preferably 1 to 2 hours. The reaction product is purified by dialysis, demineralization column, and the like, thereby to give a conjugate of phenoxyacetic acids or derivatives thereof with a high molecular compound.

[0043]

On the other hand, a mixed acid anhydride using in a mixed acid anhydride method is obtained from a reaction of carboxylic acid and haloformic ester, which is in turn reacted with a high molecular compound to produce an object conjugate of hapten-high molecular compound. This reaction is carried out in presence of base compound. A base compound includes organic bases such as triethylamine, trimethylamine, pyridine, N,N-dimethylaniline, N-methylmorpholine, DBN, DBU, DABCO; and inorganic bases such as potassium carbonate, sodium carbonate, potassium

hydrogen carbonate, sodium hydrogen carbonate. The reaction is carried out at minus 20°C to 100°C, preferably 0 to 50°C, and the reaction time is 5 minutes to 10 hours, preferably 5 minutes to 2 hours. The reaction of the mixed acid anhydride thus obtained and a high molecular compound is carried out at minus 20°C to 150°C, preferably 5°C to 100°C, more preferably 10°C to 100°C, and the reaction time is 5 minutes to 10 hours, preferably 5 minutes to 5 hours. The mixed acid anhydride method is usually carried out in a solvent. As a solvent, any solvent used commonly in a mixed acid anhydride method can be employed. Specifically, the solvent includes halogenated hydrocarbons such as dichloromethane, chloroform and dichloroethane, aromatic hydrocarbons such as benzene, toluene and xylene, ethers such as diethyl ether, dioxane, tetrahydrofuran, dimethoxyethane, esters such as methyl acetate, and ethyl acetate, and aprotic polar solvents such as N,N-dimethylformamide, dimethylsulfoxide and hexamethylphosphorictriamide. A haloformic ester used in a mixed acid anhydride method includes methyl chloroformate, methyl bromoformate, ethyl chloroformate, ethyl bromoformate and isobutyl chloroformate. The ratio of hapten and haloformic ester used in the method can be suitably chosen from a wide range.

[0044]

By a similar method to the above-described one, a compound that an enzyme is bound with phenoxyacetic acids or the derivatives thereof can be used in an immunoassay.

[0045]

Preparation of polyclonal antibody

Using a conjugate of phenoxyacetic acids or the derivatives thereof and high molecular compound, a polyclonal antibody of the present invention can be prepared by a common method. For example, the conjugate of phenoxyacetic acids-bovine serum albumin is dissolved in phosphate buffer (hereinafter referred to as PBS), Freund's complete adjuvant or incomplete adjuvant, or coadjutant such as alum is mixed therewith, the resultant mixture is used as an immunological antigen to

immunize animals. As an animal to be immunized, animals used commonly in this field can be employed, including mouse, rat, rabbit, goat, and horse.

[0046]

Administration method of immunization may be any of subcutaneous injection, intraperitoneal injection, intravenous injection, intradermal injection and intramuscular injection, and subcutaneous injection or intraperitoneal injection is preferred. Immunization can be performed once or several times in proper intervals, preferably in intervals of 1 weak to 5 weeks.

[0047]

By collecting blood from immunized animals, and then using serum separated from the blood, the presence of polyclonal antibody reacting to phenoxyacetic acids in the serum can be evaluated.

[0048]

Preparation of monoclonal antibody

Using a conjugate of phenoxyacetic acids or the derivatives thereof and high molecular compound, a monchlonal antibody of the present invention can be prepared by a common method.

[0049]

In preparation of monoclonal antibody, at least the following operations are necessary.

[0050]

(a) Preparation of conjugate of phenoxyacetic acids or the derivatives thereof and high molecular compound to use as an immunological antigen

(b) Immunization into animal

(c) Collection of blood, assay, and preparation of antibody-producing cell

(d) Preparation of myeloma cell

(e) Cell fusion of antibody-producing cell and myeloma cell

(f) Cloning of hybridoma producing an object antibody

(g) Cultivation of hybridoma or transplant to animals in order to

prepare a large amount of monoclonal antibody optionally.

(h) Measurement of reactivity of produced monoclonal antibody

Conventional method for producing hybridoma that produces monoclonal antibody is described, for example, in Hybridoma Techniques, published by Cold Spring Harbor Laboratory, 1980, and Saibousoshikikagaku (by Shuji Yamashita et. al, edited by Japan Society of Histochemistry and Cytochemistry; Gakusai Kikaku, 1986).

[0051]

Hereinafter, a preparation method of anti-phenoxyacetic acids monoclonal antibody of the present invention will be described, but it is apparent to those skilled in the art that the invention is not limited thereto.

[0052]

The processes of (a) to (c) can be conducted in almost the same manner as the method described in polyclonal antibody.

[0053]

As a myeloma cell, for example, P3/X63-Ag8 (X63) derived from Balb/c mouse (Nature, 256, 495-497 (1975)); P3/X63-Ag 8.U1 (P3U1) (Current Topics in Microbiology and Immunology, 81, 1-7 (1987)); P3/NSI-1-Ag4-1 (NS-1) (Eur. J. Immunol., 6, 511-519 (1976)); Sp2/O-Ag14 (Sp2/O) (Nature, 276, 269-270 (1978)); FO (J. Immuno. Meth., 35, 1-21 (1980)); established myeloma cell line such as MPC-11, X63.653, S194; or 210. RCY3, Ag 1.2.3 (Y3) derived from rat (Nature, 277, 131-133, (1979)) can be used.

[0054]

The established cell line described above is subcultured in Dulbecco's modified Eagle medium (DMEM) or Iscove's modified Dulbecco's medium (IMDM), so that more than 1×10^6 of cell number is acquired on the day the fusion is performed.

[0055]

Antibody-producing cells are lymphocytes, they can be generally obtained from spleen, thymic gland, lymph node, peripheral blood or combination thereof, and spleen cell is most generally used.

[0056]

After the final immunization of mouse and confirmation of production of antibody, a portion where the antibody is produced, such as spleen, is excised from the mouse to prepare spleen cells. The fusion of the spleen cell and myeloma cell obtained in process (d) can be carried out according to a known method, for example, method of Milstein et. al (Methods in Enzymology, 73, 3 (1981)). The fusion operation that is now commonly performed is a simple method employing a polyethylene glycol (PEG). PEG method is described, for example, in Saibousoshikikagaku, Shuji Yamasita et. al (ibid). Alternatively, an electric treatment (electric fusion) can be adopted appropriately (Etuko Ohkochi et. al; Experimental Medicine, 5, 1315-19, 1987). The ratio of cells used may be the same ratio as the known method, for example, the ratio of spleen cell to myeloma cell may be about 3 to 10.

[0057]

In a choice of a hybridoma population which aquired antibody-producing ability and proliferation potency by fusion of spleen cell and myeloma cell, for example, when hypoxanthine-guanine phosphoribosyltransferase deficiency strain is used as a myeloma cell strain, it can be performed by using HAT medium. Further, a portion of culture supernatant of containing the hybridoma population selected is sampled and the antibody activity to the phenoxyacetic acids is measured by ELISA described later.

[0058]

Further, cloning is preformed for the hybridoma which has been confirmed by the measurements that the antibody reacting to phenoxyacetic acids is produced. The example of this cloning includes a "limiting dilution method" where a dilution is conducted so as to contain one hybridoma in one well; a method of plating the hybridoma on soft agar medium and picking up a colony; a method of taking out one cell by a micromanipulator; and a "sorter clone method" where one cell is separated by a cell sorter. Limiting dilution method is simple and often used.

[0059]

For a well in which antibody titer has been determined, for example, by a limiting dilution method, cloning is repeated 1-4 times so as to have a stable antibody titer in the well and then a hybridoma strain that produces an anti-phenoxyacetic acids monoclonal antibody is selected.

[0060]

As a medium for culturing hybriddoma, for example, DMEM or IMDM containing fetal bovine serum is used. Hybridoma is preferably cultivated, for example, in 5 to 7% of carbon dioxide at 37°C (in an incubator in 100% humidity).

[0061]

A large scale of cultivation for production of antibody is carried out by a hollow fiber type culture apparatus. Alternatively, in an abdominal cavity of the same mouse strain (e.g. the above-described Balb/c) or Nu/Nu mouse, hybridoma can be proliferated to produce an antibody from the ascites fluid.

[0062]

Thus obtained culture supernatant or the ascites fluid can be used as anti-phenoxyacetic acids monoclonal antibody. It can be further subjected to dialysis, salting-out with ammonium sulfate, gel filtration, freeze dry and the like, and then antibody-containing fractions are collected and purified to give an anti-phenoxyacetic acids monoclonal antibody. If further purification is required, by employing known methods such as ion-exchange column chromatography, affinity chromatography, open column chromatography and high performance liquid chromatography (HPLC), operations for collecting the antibody-containing fractions are performed once or in a plurality of operations.

[0063]

The anti-phenoxyacetic acids monoclonal antibody thus obtained can be determined for subclass and antibody titer by a known method such as ELISA described later.

[0064]

Measurement of phenoxyacetic acids by antibody

A method of measuring phenoxyacetic acids by antibody employed in the present invention includes various methods used generally for detecting antigen such as radioimmunoassay (RIA), ELISA (Engvall, E., Meth. Ensymol., 70, 419-439 (1980)), a fluorescence antibody technique, a plaque technique, a spot technique, hemagglutination, and Ouchterlony ("Hybridoma method and monoclonal antibody," published by Kabushiki Kaisha R&D Planning, page 30-53, March 5, 1982). The ELISA method is widely used in view of sensitivity and simplicity.

[0065]

The above measurements, for example, indirect competition inhibition ELISA, can be conducted in the following steps:

(a) Firstly, conjugate of phenoxyacetic acids or the derivatives being an antigen and high molecular compound is immobilized on a support to form a solid phase.

(b) Surface of the solid phase on which antigen does not adhere is blocked by a substance irrelevant to antigen, for example, protein.

(c) A sample containing various concentrations of phenoxyacetic acids and an antibody are added thereto, followed by competitively reacting the antigen with the above immobilized antigen and free phenoxyacetic acids to produce an immobilized antigen-antibody complex, and a free phenoxyacetic acids-antibody complex.

(d) The amount of free phenoxyacetic acids in the sample can be determined by measurement of the amount of the immobilized antigen-antibody complex using a calibration curve predetermined.

[0066]

In the step (a), as a support for immobilizing an antigen, it is not particularly limited, a support commonly used in ELISA can be employed. For example, a 96-well microtiter plate made of polystyrene is included.

[0067]

To immobilize an antigen on a support, for example, a buffer containing the antigen is laid on the support and incubated. As a buffer, a

known buffer such as Dulbecco's phosphate buffer can be used. The concentration of antigen in buffer can be selected from a wide range, normally 0.01 to 100 μg/ml, and preferably 0.05 to 10μg/ml. When 96-well microtiter plate is used as a support, it is not more than 300 μl/well, and preferably about 50 to 150μl/well. Further, the condition of incubation is not particularly limited, it is suitable to be normally at about 4°C overnight.

[0068]

Regarding blocking in the step (b), because in a support on which the conjugate of phenoxyacetic acids or the derivatives and high molecular compound is immobilized, there is a portion where not only phenoxyacetic acids or the derivatives but also an antibody to be added later may be adsorbed, the blocking is performed to prevent such adsorption. As a blocking agent, for example, bovine serum albumin (BSA) or skim milk solution can be used. Alternatively, as a blocking agent, a commercially available blocking agent, such as Block Ace, code No. UK-25B manufactured by Snow Brand Milk Products Co., Ltd. can be used. Specifically, it is no limited, for example, a suitable amount of buffer containing a blocking agent (e.g. 85 mM borate buffer (pH 8.0) supplemented with 1% BSA and 60 mM NaCl) is added to the portion where an antigen has been immobilized, incubated at about 4°C overnight, and then washed with the buffer. As a washing buffer, it is not particularly limited, for example, a borate buffer to which 60 mM NaCl is supplemented is suitable.

[0069]

Next, in the step (c), an immobilized antigen is contacted with a sample containing phenoxyacetic acids and an antibody, thereby the antibody is reacted with the immobilized antigen and free phenoxyacetic acids to produce an immobilized antigen-antibody complex and a free phenoxyacetic acids-antibody complex.

[0070]

In this case, an antibody against the phenoxyacetic acids of the present invention is added as a first antibody, and further, an antibody against the first antibody, which is bound to a label enzyme, is sequentially

added thereto as a second antibody.

[0071]

The first antibody is dissolved in buffer before addition. The reaction is carried out at about 25°C for about one hour, although it is not limited thereto. After the reaction, a support is washed with buffer to remove the unreacted first antibody. As a washing buffer, for example, borate buffer to which 60 mM NaCl is supplemented is preferred.

[0072]

Next, the second antibody is added thereto. For example, when mouse monoclonal is used as a first antibody, it is suitable to use an anti-mouse antibody-goat antibody bound to an enzyme (e.g. peroxidase or alkali phosphatase). It is desirable that the first antibody bound on support is reacted with a second antibody having a final absorbance of 4 or less, preferably 0.5 to 3.0 by dilution. A buffer is used for dilution. The reaction is carried out at about 25°C for about one hour, although it is not limited thereto. After the reaction, the reaction mixture is washed with buffer. By the above reaction, the second antibody is bound to the first antibody. A labeled first antibody may be used, in such case, a second antibody is not required.

[0073]

Next, a solution containing color-producing substrate that can react with an enzyme of a second antibody bound on support in the step (d) is added thereto, the amount of phenoxyacetic acids can be determined by a calibration curve.

[0074]

When peroxidase is used as an enzyme to be bound to a second antibody, for example, as a color-producing substrate, hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine are used. It is not limited to them, a color-producing substrate solution is added and allowed to react at room temperature for about 10 minutes, then the enzyme reaction is terminated by addition of 1N sulfuric acid. In the case of using 3,3',5,5'-tetramethylbenzidine, absorbance is measured at 450 nm. On the

other hand, when alkali phosphatase is used as an enzyme to be bound to a second antibody, for example, suitably, p-nitrophenyl phosphate that is used as a substrate is color-developed, and the enzyme reaction is terminated by addition of 2N NaOH to measure absorbance at 415 nm.

[0075]

According the above-described indirect competitive inhibition ELISA, by using monoclonal antibody TCA28-50 of the present invention, the amount of 2,4,5-T can be measured in a range of 0.001 to 10 ng/ml, preferably 0.04 to 1.25 ng/ml. By using TCA28-5 and TCA31-4, the amount of 2,4,5-T can be measured in a range of 1 ng/ml to 10 μg/ml, preferably 20 to 1250 ng/ml (see Example 5, Fig. 1).

[0076]

Alternatively, measurement of phenoxyacetic acids, for example can be performed by a direct competitive inhibition ELISA using a monoclonal antibody of the present invention as described below.

[0077]

(a) First, a monoclonal antibody of the present invention is immobilized on a support to form a solid phase.

[0078]

(b) Support surface on which antibody is not immobilized is blocked by a substance irrelevant to antigen, for example, protein.

[0079]

(c) Aside from the above steps, an enzyme-linked hapten that is phenoxyacetic acids or the derivatives bound to the enzyme is added to a sample containing phenoxyacetic acids in various concentrations to prepare a mixture.

[0080]

(d) The above mixture is reacted with the above-described antibody immobilized on the support.

[0081]

(e) The amount of phenoxyacetic acids in a sample is determined by measuring an immobilized antibody-enzyme-linked hapten complex using a

calibration curve predetermined.

[0082]

In the step (a), a support on which the monoclonal antibody is immobilized is not particularly limited, a support commonly used in ELISA can be employed, for example, including 96-well microtiter plate. For immobilization of the monoclonal antibody, for example, a buffer containing the monoclonal antibody is laid on the support, and incubated. The composition and concentration of buffer used can be the same as that in the above indirect competitive inhibition ELISA.

[0083]

Regarding blocking in the step (b), because, in a support on which the monoclonal antibody is immobilized, there is a portion where phenoxyacetic acids and enzyme-linked hapten in a sample that is added later may be adsorbed in irrelevant to an antigen-antibody reaction, the blocking is performed to prevent such adsorption. Blocking agent and the method to be used can be the same as that in the above indirect competitive inhibition ELISA.

[0084]

The preparation of enzyme-linked hapten used in the step (c) is not particularly limited, a method of binding an enzyme to phenoxyacetic acids or the derivatives is employed. For example, the activated ester method described above can be adopted. The enzyme-linked hapten thus prepared is mixed with a sample containing phenoxyacetic acids.

[0085]

In the step (d), the mixture is contacted with antibody immobilized on support, thereby competition inhibition reaction of phenoxyacetic acids and enzyme-linked hapten in the mixture is caused to produce a complex of them and an immobilized antibody. A sample containing phenoxyacetic acids is used by diluting with a suitable buffer. The reaction is carried out at about 25°C for about one hour, although it is not limited thereto. After the reaction, the support is washed with a buffer to remove the unreacted enzyme-linked hapten. A washing buffer can employ, for example, 85 mM

borate buffer (pH 8.0) to which 60 mM NaCl is supplemented.

[0086]

Further, in the step (e), a solution containing a color-producing substrate that can react with enzyme-linked hapten is added in the same manner as the above-described indirect competitive inhibition ELISA, the amount of phenoxyacetic acids can be calculated by measuring absorbance and using a calibration curve.

[0087]

TCA28-50, one of the monoclonal antibodies of the present invention, has a difference in measurement sensitivity to 2,4,5,-T depending on various enzyme-linked haptens added to the measurement system in the direct competitive inhibition ELISA. Namely, for example, in the case of using a conjugate of 2,4,5-T and peroxidase as an enzyme-linked hapten, the amount of 2,4,5-T can be measured in a range of 1 to 500 ng/ml, preferably 5 to 200 ng/ml; in the case of using a conjugate of 2,4,5-T derivative and peroxidase, it can be measured in 10 to 1000 ng/ml, preferably 12.5 to 400 ng/ml; and in the case of using conjugate of mecoprop and peroxidase, it can be measured in 0.5 to 20 ng/ml, preferably 1 to 10 ng/ml (see Example 8, Fig.2).

[0088]

In the indirect competitive inhibition ELISA described above, in the same manner as the direct competitive inhibition ELISA, various phenoxyacetic acids or the derivatives can be used as an antigen to be immobilized on a support.

[0089]

Methanol resistance of antibody of the present invention

By using monoclonal antibody TCA28-50, which is an embodiment of the present invention, 2,4,5-T can be recognized in concentration dependence; according to the indirect competitive inhibition ELISA in presence of methanol of 0% to 40%, preferably 10% to 40%; according to the direct competitive inhibition ELISA, in presence of methanol of 0 to 10%. Phenoxyacetic acids are soluble easily in organic solvents. By considering

that the analysis is generally performed in organic solvents such as methanol, such characteristics of the monoclonal antibody of the present invention is very effective.

[0090]

Cross-reactivity of antibody of the present invention

By the above-described indirect competitive inhibition ELISA and direct competitive inhibition ELISA, the cross-reactivity of monoclonal antibody TCA28-50 of the present invention can be examined. TCA28-50 exhibits a strong reactivity to 2,4,5-T and trichlopyr as well, but it exhibits almost no cross-reactivity to other phenoxyacetic acids. For example, in the indirect competitive inhibition ELISA, a cross-reactivity to 2,4-D, 2,4-DB, fluroxypyr or MCP is about one four hundredth or less based on that to 2,4,5-T; and also in the direct competitive inhibition ELISA, the cross-reactivity to 2,4-D is about one sixtieth or less based on that to 2,4,5-T (see Example 10, Table 2).

[0091]

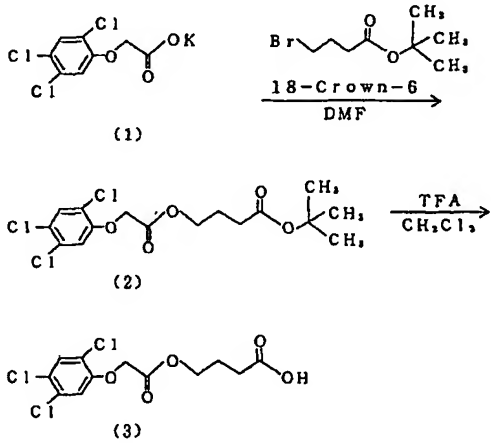
Hereinafter, the examples are described to explain the present invention, but the examples are not intended to limit the technical scope of the present invention. From the description in the present specification, those skilled in the art may easily give modification and change thereto, these are included in the technical scope of the invention.

[0092]

[Examples]

Example 1 Synthesis of 2,4,5-T derivatives

[Compound 12]



Synthesis of (2)

First, 0.9 g (4.0 mmol) of potassium 2,4,5-trichlorophenoxyacetate (1) is dissolved in 10 ml of DMF, then 1.5 g (5.1 mmol) of tert-butyl 4-bromobutyrate and 1.3 g (4.9 mmol) of 18-crown-6 were added thereto, followed by stirring at room temperature overnight. The reaction mixture was extracted for partition with water-ether (100 ml, 3 times), the combined ether solution was dried over anhydride magnesium sulfate, and concentrated under reduced pressure. The concentrate was purified through a silica gel chromatography (hexane:ethyl acetate=4:1) to give (2) of 1.3 g (yield 81%) as a white crystal.

[0093]

Synthesis of 2,4,5-T derivative (3)

First, 1.5 g (3.8 mmol) of ester (2) was dissolved in 50 ml of dichloromethane, then 5 ml of trifluoroacetic acid was added thereto, followed by stirring at room temperature for 1.5 hours. The reaction mixture was azeotroped with dichloromethane until pungent odor of trifluoroacetic acid was vanished. The concentrate was dissolved in a small amount of ethyl acetate, and hexane was added thereto, thereby (3) of 1.0 g (yield 78%) was crystallized (referred to as 2,4,5-T derivative) as a white crystal.

[0094]

[Table 1]

^{1}H-NMR ($CDCl_3$ 2.02 ppm (q,2H), 2.43 ppm (m,2H), 4.29 ppm (t,2H), 4.69 ppm (s,2H), 6.96 ppm (s,1H), 7.49 ppm (s,1H)

[0095]

Example 2: Binding of 2,4,5-T or 2,4,5-T derivative with carrier protein by activated ester method

First, 3.5 μmol of 2,4,5-T and 2,4,5-T derivative synthesized in Example 1 as hapten were each dissolved in 50 μl of dimethyl sulfoxide (hereinafter abbreviated as DMSO). Next, to these solutions, N-hydroxysuccinimide (5 μmol) dissolved in 10 μl of DMSO was added, then 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (4 μmol)

dissolved in 20 µl of DMSO was added thereto. The reaction was carried out at room temperature for 1.5 hours, then 10 mg of bovine serum albumin (hereinafter abbreviated as BSA) or keyhole limpet hemocyanin (hereinafter abbreviated as KLH) each dissolved in 500 µl of 85 mM borate buffer (pH 8.0) was added to this reaction mixture, again the reaction was carried at room temperature for 1.5 hours. After the reaction, the reaction mixture was dialyzed against Dulbecco's phosphate buffer (hereinafter abbreviated as PBS (-)) to each prepare a conjugate of 2,4,5-T and KLH, a conjugate of 2,4,5-T and BSA, a conjugate of 2,4,5-T derivative and KLH, and a conjugate of 2,4,5-T derivative and BSA.

[0096]

Example 3 Immunization of conjugate of 2,4,5-T and KLH, and conjugate of 2,4,5-T derivative and KLH

Balb/c mouse was used for immunization. Each 100 µg of the conjugate of 2,4,5-T and KLH or the conjugate of 2,4,5-T derivative and KLH prepared in Example 2 was dissolved in 50µl of PBS (-), and mixed with the equal amount of Freund's complete adjuvant into emulsion. The resultant emulsion was inoculated in the abdominal cavity of mouse. One month later, 1/4 of the amount of emulsion used in primary immunization was boosted. The two months later from the boost for the conjugate of 2,4,5-T and KLH, and three weeks later for the conjugate of 2,4,5-T derivative and KLH, final immunization was performed respectively with the equal amount of boost.

[0097]

Example 4 Preparation of monoclonal antibody

Cell fusion was performed using spleen cell from mouse experienced 3 days after the final immunization in Example 3. The spleen cells were taken out in DMEM, then washed with DMEM three times while removing large solids using a stainless steel mesh, and mixed with myeloma cell of mouse P3-X63-Ag8.653 in a cell number ratio of 5:1 (spleen cell : myeloma cell). The mixture was centrifuged at 1,200 rpm for 5 minutes to collect the cell precipitates. To the cell precipitates, 1 ml of 50% polyethylene glycol

(molecular weight of 1,500) preheated to 37°C was added to fuse cells. The cell fusion was terminated by adding 10 ml of DMEM slowly, and further adding 1 ml of fetal bovine serum (hereinafter abbreviated as FBS). The fusion cell was suspended in HAT medium that is DMEM added with 10% FBS further supplemented with hypoxanthine (100 μM), aminopterin (40 μM) and thymidine (16 μM), and then divided into 96-well of polystyrene plate in an amount of 2×10^5 cells/well, cultivated at 37°C in presence of 5% carbon dioxide for 10-14 days. After cultivation, the presence of antibody activity in the well was each screened.

[0098]

The antibody activities were measured by ELISA using the conjugate of 2,4,5-T and BSA or the conjugate of 2,4,5-T derivative and BSA prepared in Example 2. The conjugate of BSA and a corresponding immunogen dissolved in PBS (-) and BSA (4 μg/ml) were added in the 96-well microtiter plate each in an amount of 100 μl/well, and allowed to stand at 4°C overnight for immbilization. Next, the solution in each well was replaced by 300 μl/well of a buffer containing a blocking agent (85 mM borate buffer (pH 8.0) supplemented with 1% BSA and 60 mM NaCl), and blocked at room temperature for 1 hour. After the well was washed with a washing buffer (borate buffer added with 60 mM NaCl), then 100μl/well of culture supernatant of hybridoma was added thereto, and allowed to react at room temperature for 1 hour. After the well was washed with a washing buffer three times, peroxidase-labeled anti-mouse IgG antibody (Cappel Products) diluted 1000 times with a dilution buffer for second antibody (85 mM borate buffer (pH 8.0) added with 0.3% BSA and 60 mM NaCl) was added in an amount of 100 μl/well, and allowed to react at room temperature for 1 hour. After the well was washed with a washing buffer three times, the well was color-developed for ten minutes by adding thereto a solution containing peroxidase substrate (0.1 M sodium acetate buffer (pH 5.5) added with 3.3' 5,5'-tetamethylbenzidine (100 μg/ml) and 0.006% hydrogen peroxide), and after the reaction was terminated with 1N sulfuric acid, then absorbance was measured at 450 nm.

[0099]

The hybridoma exhibiting antibody activity in well was cloned by limiting dilution method and selected as a hybridoma producing a monoclonal antibody.

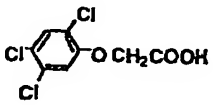
[0100]

From these results as shown in Table 1, a hybridoma producing the monoclonal antibody that reacts with the conjugate of 2,4,5-T and BSA or the conjugate of 2,4,5-T derivative and BSA while not react to BSA was each separated into one strain (TCA28-50) and two strains (TCA28-5 and TCA31-4).

[0101]

[Table 2]

Table 1 Separated hybridoma

Structure of hapten used	Hybridoma	Subclass of monoclonal antibody	Reactivity to conjugate of hapten and BSA	Reactivity to BSA
	TCA 28-50	IgG2a	+	–
	TCA 28-5	IgG1	+	–
	TCA 31-4	IgG1	+	–

[0102]

Among them, TCA28-50 was deposited September 11, 1996 in accession number; FERM P-15848 in National Institute of Bioscience and Human Technology (zip: 305, 1-3, Higashi 1-chome, Tukuba, Ibaragi).

[0103]

Example 5 Examination on reactivity of monoclonal antibody to 2,4,5-T by indirect competitive inhibition ELISA

Reactivities of the monoclonal antibodies produced by three strains that were obtained in Example 4 (hereinafter, monoclonal antibodies are named the same name as the hybridomas by which the antibodies are

produced) to 2,4,5-T were examined by the indirect competitive inhibition ELISA.

[0104]

First, for TCA28-50, a conjugate of 2,4,5-T and BSA (0.5 μg/ml); for TCA28-5 and TCA31-4, a conjugate of 2,4,5-T derivative and BSA (2 μg/ml) was each immobilized on 96-well microtiter plate in the same manner as ELISA shown in Example 4. Next, the solution in each well was replaced by 300μl/well of a buffer containing a blocking agent, and blocked at room temperature for 1 hour. After the well was washed, the solution containing 2.4.5-T which was diluted into an appropriate concentration with dilution buffer (85 mM borate buffer (pH 8.0) added with 150 mM NaCl) was added thereto in an amount of 50 μl/well, then immediately, the solution containing the antibody which was diluted in an appropriate concentration with the same dilution buffer was added thereto in an amount of 50 μl/well, and allowed to react at room temperature for 1 hour. After the well was washed three times, a peroxidase labeled anti-mouse IgG antibody (Cappel Products) diluted 1000 times with a dilution buffer for second antibody was added in an amount of 100 μl/well, and allowed to react at room temperature for 1 hour. After the well was washed three times, the well was color-developed in the same manner as ELISA in Example 4, and measured for absorbance at 450 nm.

[0105]

From these results as shown in Fig.1, TCA28-50 reacted with 2,4,5-T in a measurement range of about 0.04 to 1.25 ng/ml, TCA28-5 and TCA31-4 in that of about 20 to 1250 ng/ml. Therefore, all of these antibodies react with 2,4,5-T, among them, it was found that TCA28-50 reacts with 2,4,5-T with the highest sensitivity.

[0106]

Example 6 Purification of monoclonal antibody

Purification of antibody was performed for TCA28-50 that reacted with 2,4,5-T with the highest sensitivity. First, to the culture supernatant that hybridoma was cultured using DMEM supplemented with 10% FBS,

ammonium sulfate was added so as to be 50% saturation, followed by stirring at 4°C overnight. Distilled water was added to the precipitate to solubilize, then, the resultant mixture was dialyzed against 5 mM phosphate buffer (pH 7.0), and purified through DEAE cellulose column chromatography.

[0107]

Example 7 Preparation of peroxidase labeled hapten

First, 1.25 μmol of each 2,4,5-T, 2,4,5-T derivative and mecoprop that is an analogue herbicide were dissolved in 100 μl of DMSO. To the solution, 3 μl (5.5 μmol) of N-hydroxysuccinimide and 7 μl (3.5 μmol) of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide were added, and allowed to react at room temperature for 1 hour. To the reaction mixture, 40 μl of 1M aqueous sodium hydrogen carbonate was added, further 500 μl of a solution containing peroxidase (20 mg/ml) was added thereto, mixed and allowed to react at room temperature for 3 hours. The resultant reaction mixture was subjected to a gel filtration column (Sephadex G-25) to remove low molecular compounds, thereby to give a peroxidase labeled hapten.

[0108]

Example 8 Examination on reactivity of monoclonal antibody to 2,4,5-T by direct competitive inhibition ELISA

Direct competitive inhibition ELISA was carried out in the following steps: The TCA28-50 purified in Example 6 was dissolved in 50 mM sodium carbonate buffer (pH 9.6) at a concentration of 10 μg/ml, and the mixture was placed in a 96-well microtiter plate in an amount of 100 μl/well, and allowed to stand at 4°C overnight for immobilization. On the other hand, the solution containing 2,4,5-T diluted with a dilution buffer added with 0.15% BSA in an appropriate concentration and the peroxidase labeled hapten were mixed. After blocking, this solution was added to the well in an amount of 100 μl/well and allowed to react at room temperature for 1 hour. After washing with washing buffer five times, the well was color-developed in the same manner as ELISA in Example 4, and measured for absorbance at 450 nm.

[0109]

From these results ase shown in Fig.2, the difference was occurred in the measurement sensitivity of 2,4,5-T depending on peroxidase labeled hapten used. Namely, as a peroxidase labeled hapten, in the case of using the conjugate of 2,4,5-T and peroxidase, it reacted to 2,4,5-T in a range of about 5 to 200 ng/ml; in the case of using the conjugate of 2,4,5-T derivative and peroxidase, in about 12.5 to 400 ng/ml; and in the case of using the conjugate of mecoprop and peroxidase, in about 1 to 10 ng/ml.

[0110]

Thus, also in the direct competitive inhibition ELISA, TCA28-50 reacted with 2,4,5-T, in the case of competition with the conjugate of mecoprop and peroxidase, TCA28-50 reacted with 2,4,5-T with the highest sensitivity.

[0111]

Example 9 Methanol resistance of monoclonal antibody TCA28-50

On monoclonal antibody TCA28-50, using indirect competitive inhibition ELISA shown in Example 5 and direct competitive inhibition ELISA shown in Example 8, the influence of methanol was examined on these measurement systems. Methanol was added to a solution for competition reaction at a concentration of 0 to 60% for the indirect competitive inhibition ELISA, and at 0 to 20% for the direct competitive inhibition ELISA. The results in the indirect competitive inhibition ELISA are shown in Fig. 3 and the results in the direct competitive inhibition ELISA are shown in Fig. 4. From the results, in the indirect competitive inhibition ELISA, although it is observed that the measurement sensitivity was lowered by the increase in methanol concentration, 2,4,5-T was able to be measured in a solution containing up to at least 40% methanol. On the other hand, in the direct competitive inhibition ELISA, although decrease in absorbance was observed, 2,4,5-T was able to be measured in a solution containing up to 10% methanol with almost the same measurement sensitivity.

[0112]

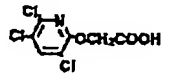
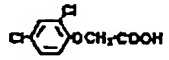
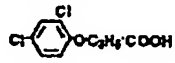
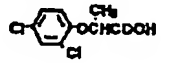
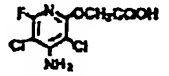
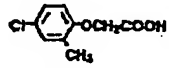
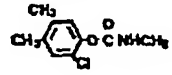
Example 10 Cross-reactivity of monoclonal antibody TCA28-50 to analogous compounds of 2,4,5-T

Using indirect competitive inhibition ELISA shown in Example 5 and direct competitive inhibition ELISA shown in Example 8, cross-reactivity of TCA28-50 to the analogous compounds of 2,4,5-T was examined. The results are shown in Table 2 as the respective IC_{50} values that is the concentrations of a compound which inhibits the reaction by 50% based on the reaction with no addition of compound.

[0113]

[Table 3]

Table 2 Examination on cross-reactivity of TCA28-50 to 2,4,5-T and the related compounds

Name of pesticide	Chemical structure	IC_{50} (ng/ml) Indirect competitive inhibition ELISA	IC_{50} (ng/ml) Direct competitive inhibition ELISA
2,4,5-T		0.23	3.5
Trichlopyr		0.36	4.0
2,4-D		110	230
4-CPA		4000	5800
2,4-DB		230	2700
Dichlorprop		2600	6900
Fluroxypyr		360	1100
MCP		320	1500
MCPB		1000	6200
Mecoprop		11000	12000
Carbanolate		5200	7100

[0114]

As seen from Table 2, TCA28-50 exhibited a strong reactivity to 2,4,5-T and trichlopyr as well, but did almost no strong reactivity to other phenoxyacetic acids. For example, in indirect competitive inhibition ELISA, a cross-reactivity to 2,4-D, 2,4-DB, fluroxypyr and MCP is about one four hundredth or less based on that to 2,4,5-T; and also in the direct competitive inhibition ELISA, the cross-reactivity is about one sixtieth or less based on that to 2,4,5-T.

[Brief Description of the Drawings]

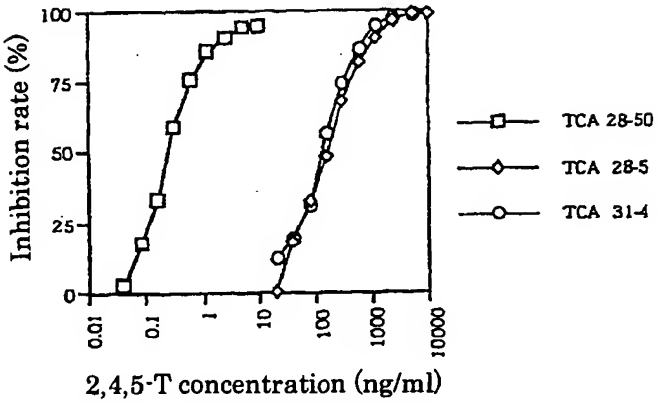
Fig. 1 shows the results of measurement sensitivity of respective monoclonal antibodies of the present invention to 2,4,5-T measured by indirect competitive inhibition ELISA.

Fig. 2 shows the results of measurement sensitivity of monoclonal antibody TCA 28-50 of the present invention to 2,4,5-T measured by direct competitive ELISA competition method.

Fig. 3 shows the influence of methanol on indirect competitive inhibition ELISA using monoclonal antibody TCA 28-50 of the present invention.

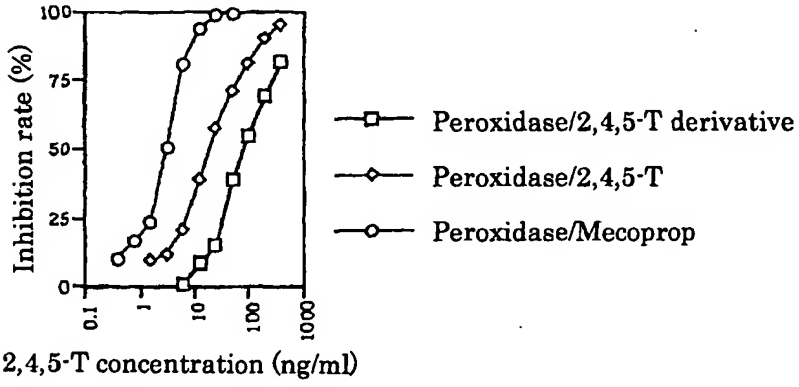
Fig. 4 shows the influence of methanol on direct competitive inhibition ELISA using monoclonal antibody TCA 28-50 of the present invention.

[Fig. 1]



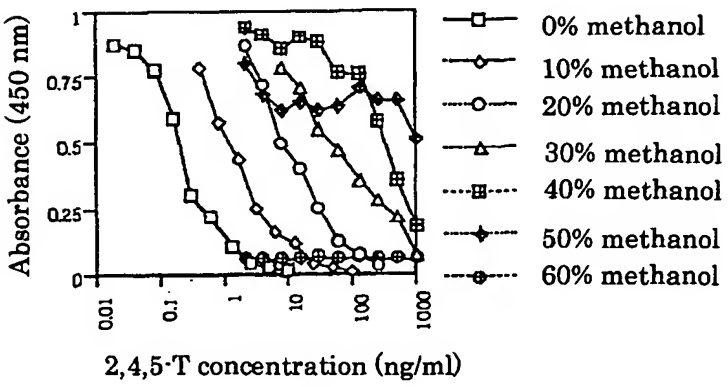
Measurement sensitivity of respective monoclonal antibodies to 2,4,5-T (Indirect competitive inhibition ELISA)

[Fig.2]



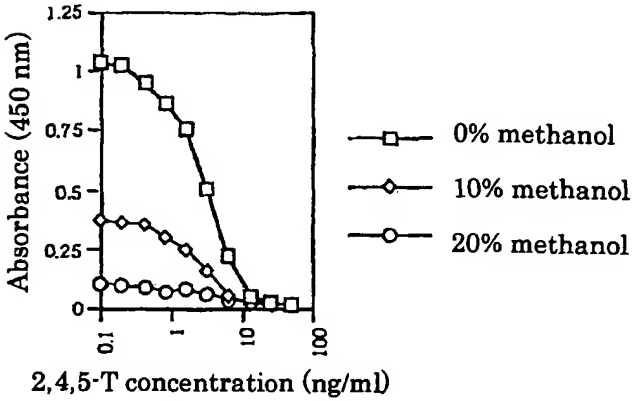
Change of measurement sensitivity of TCA 28-50 to 2,4,5-T depending on different peroxidase conjugates
(Direct competitive inhibition ELISA)

[Fig. 3]



Influence of methanol on indirect competitive inhibition ELISA

[Fig.4]



Influence of methanol in direct competitive inhibition ELISA